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## Dcp1a phosphorylation along neuronal development and stress

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The authors would like to dedicate this paper to the late Prof. Irith Ginzburg, who inspired this work and was our beloved mentor for many years.

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### ABSTRACT

**Decapping protein 1a (Dcp1a) is found in P-bodies and functions in mRNA cap removal prior to its degradation. The function and binding partners of Dcp1a have been thoroughly studied, however its expression pattern is still unclear. In this study we have monitored Dcp1a expression along brain development, neuronal differentiation and during cellular stress. We found that Dcp1a is hyperphosphorylated under these physiological conditions. We followed our observations and identified the specific amino acid residues that are phosphorylated. These findings suggest a novel post-translational modification that may influence the function of Dcp1a in response to various physiological cues.**

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### 1. Introduction

Regulation of mRNA degradation is a key step in the control of gene expression [1]. In budding yeast, *Saccharomyces cerevisiae* mRNAs are degraded via two pathways. In both pathways, removal of the poly(A) tail by deadenylation is the initial step. After deadenylation, removal of the 5'-cap structure by decapping enzymes occurs and proceeds via 5'-3' digestion of the transcript by the exonuclease Xrn1. Alternatively, mRNAs can be degraded from 3' to 5' by the exosome, a complex of several nucleases [2]. Messenger RNA molecules that harbor premature-termination codons, are subjected to rapid degradation via the non-sense mediated decay (NMD) pathway. In this pathway mRNAs can go through accelerated deadenylation followed by 3'-5' exonucleolytic activity or deadenylation-independent decapping, followed by 5'-3' cleavage [3,4].

Decapping is thus a key step in the 5'-3' decay pathway because it initiates the degradation of both normal and aberrant mRNAs [5].

In this process the 5'-cap is cleaved by specific enzymes to release a 7-methyl GDP product. Studies in *S. cerevisiae* identified two interacting proteins, decapping protein 1p (Dcp1p) and Dcp2p which are involved in mRNA decapping [6–8]. Dcp1p was also suggested to be a phosphoprotein, however the locations and role of its phosphorylation was not determined [7]. In humans, Dcp1p and Dcp2p homologs, termed hDcp1a, hDcp1b, and hDcp2 were identified. hDcp1a and hDcp2 interact with each other in an RNA independent manner, and copurify with decapping activity in vitro [9]. Another study suggested that Dcp1a also termed as SMAD4-interacting transcription factor (SMIF), interacts with SMAD4 protein and acts as a transcription co-activator in the transforming growth factor  $\beta$  (TGF- $\beta$ ) pathway. Upon TGF- $\beta$  activation, SMIF-SMAD4 complexes are found in the nucleus, and initiates the expression of TGF- $\beta$ /bone morphogenic protein (BMP) regulated genes [10].

In this study we investigated the expression of Dcp1a using fluorescence microscopy and SDS-PAGE analysis. We found that Dcp1a migrates as two distinct bands in different cell types and that the upper band is a phosphorylated form of the protein. In addition, arsenate treatment of 293 HEK cells, induced hyperphosphorylation of Dcp1a. When specific mutations were introduced in the protein sequence, the phosphorylation of Dcp1a was significantly reduced. Our results suggest a possible link between

Abbreviations: Dcp1a, decapping protein 1; TGF- $\beta$ , transforming growth factor  $\beta$ ; BMP, bone morphogenic protein; SMIF, SMAD4-interacting transcription factor

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physiological changes, Dcp1a phosphorylation, and RNA regulation.

## 2. Materials and methods

### 2.1. Plasmids and antibodies

Polyclonal antibody against Dcp1a was a generous gift from Jens Lykke-Andersen and Bertrand Seraphin [11]. YFP-Dcp1a construct and TIAR antibodies were a gift from Nancy Kedersha [12].

### 2.2. Tissue culture, drug treatments and transfections

P19 cells were grown in minimal essential medium (MEM, Biological Industries Beit-Haemek, Israel), supplemented with 5% heat-inactivated fetal calf serum in a humidified 5% CO<sub>2</sub> incubator at 37 °C. P19ND (non-differentiated) Cells at day 0 (D0) were induced to differentiate in the following manner:  $1 \times 10^6$  cells were seeded into a 90 mm petri dish in 10 ml of MEM-5%-FCS medium and incubated with 1 mM all-trans retinoic acid (RA) (Sigma-Aldrich, Rehovot, Israel) for 2 days in a humidified incubator. Cell aggregates were then pelleted gently and fresh medium and RA was added. After two more days of aggregation (D4), cells were pelleted gently and pellet washed once with PBS. Pellet was then incubated with trypsin (Biological Industries) for 1 min. and mechanically disrupted with 2 ml of DMEM-2.5%-FCS into single cell suspension. Cells were plated on poly-L-lysine covered tissue culture plates and incubated for 24 h. Cytosine arabinoside (Sigma-Aldrich) was added (D5) to a final concentration of 5 mM and cells left in the incubator for three more days, when a definite neuronal phenotype was observed (D8).

HEK-293 cells were grown in Dulbecco's modified Eagle's medium (DMEM; GIBCO BRL Company; Grand Island, USA), supplemented with 10% (v/v) fetal calf serum Biological Industries), 2 mM glutamine, 50 units/ml penicillin, and 50 µg/ml streptomycin. In order to induce cellular stress response, cells were treated with 1 mM sodium arsenate for 30 min (Sigma-Aldrich). 293 HEK cells were transfected using the calcium phosphate precipitation method as described previously [13].

### 2.3. Brain homogenization and immunoblotting

C57/Black mice were anesthetized (ketamine) and decapitated. Brains were dissected and then homogenized in 10-times volume of buffer H (20 mM HEPES pH 7.4, 0.3 M sucrose, 1 mM EDTA supplemented with 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 µg/ml pepstatin, 1 mM phenylmethylsulfonylfluoride) by 15 strokes in glass-teflon homogenizer. Protein levels were determined using Bradford reagent. Equal amounts of protein were loaded on 10% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked in nonfat milk for 2 h, incubated with primary antibodies, as indicated over night at 4 °C, washed and incubated with horseradish peroxidase-conjugated secondary antibody for 1 h. Membranes were then developed with enhanced chemiluminescence.

### 2.4. Immunostaining of fixed cells

Cells were grown on coated coverslips with poly-L-lysine. Cells were fixed with 4% paraformaldehyde in 4% sucrose for 20 min at room temperature as previously described [14]. Following permeabilization with 0.3% Triton X-100 for 3 min, and blocking with blocking buffer (10% goat serum, 2% bovine serum albumin (BSA), 1% glycine, and 0.1% Triton (Sigma-Aldrich) in phosphate-buffered saline (PBS)), the slides were incubated with primary

antibodies as indicated for 1.5 h at room temperature, washed and reacted with a secondary antibody conjugated to fluorescein isothiocyanate (FITC) or Cy3 as necessary. The slides were visualized by confocal laser microscopy using 488 nm and 545 nm laser excitations, for FITC and Cy3, respectively.

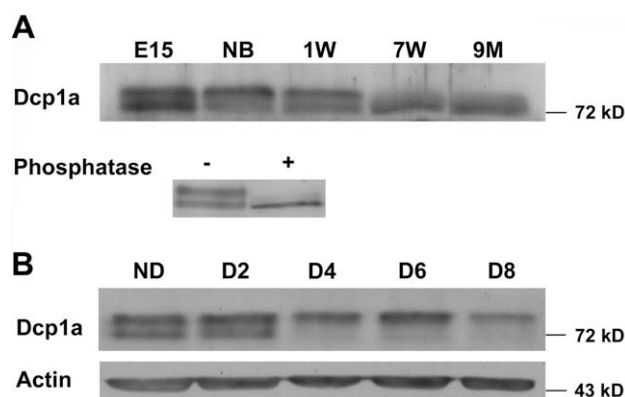
### 2.5. Site-directed mutagenesis

The mutations S315A, S319A and S321A were introduced into a YFP-Dcp1a construct using the QuikChange Site-Directed Mutagenesis Kit by Stratagene as specified by the manufacturer. The following primers were used (from the 5' to 3' end): CCTACA-CAATCCCGTTGGCCCTGTTCTCAGTCC and GGACTGAGAACAGG-GGCCAACGGGATGTGTAGG for S315A; CGTTGAGCCCTGTTCTC-GCTCCCACTCTGCCAGC and GCTGGCAGAGTGGGAGCGAGAACA-GGGCTCAACG for S319A; GCCCTGTTCTCAGTCCCGCTCTGCCAGCT-GAAGC and GCTTCAGCTGGCAGAGCGGGACTGAGAACAGGGC for S321A; CCTACACAATCCCGTTGGCCCTGTTCTCCTCCCGCTCTGC-CAGC and GCTGGCAGAGCGGGAGCGAGAACAGGGGCCAACGGG-ATTGTGTAGG for S315A/S319A/S321A.

## 3. Results

### 3.1. Dcp1a is phosphorylated along neuronal development

We followed Dcp1a expression in brain homogenates from mice at different ages. We found that Dcp1a migrates as two bands and that the relative intensity of the bands changes during mouse development (Fig. 1A). This pattern of expression is common in proteins that undergo post-translational modifications, such as phosphorylation. Therefore, we treated a protein sample taken from E15 mouse brain with phosphatase prior to the Western-blot analysis. The treated Dcp1a migrated as a single lower band as compared to a non-treated control. This observation supports our initial assumption that Dcp1a upper band is a phosphorylated form of the protein. We used another cell model for neuronal differentiation, P19 embryonic carcinoma (EC) cells, and monitored Dcp1a expression during neuronal differentiation. These cells are cultured in suspension while treated with retinoic acid (RA) for 4 days. After 4 days the cells are plated, and by day 8 (post RA treatment) a clear neuronal phenotype is visible. We analyzed Dcp1a expression in

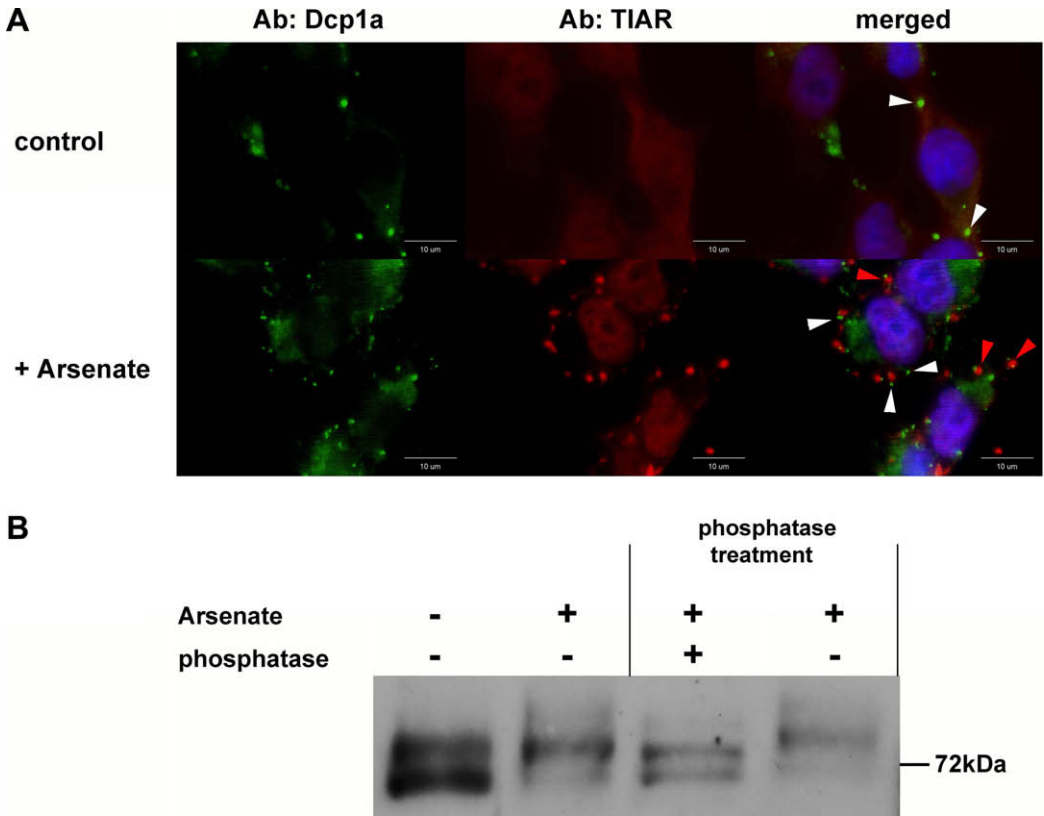


**Fig. 1.** Dcp1a expression and phosphorylation levels along brain development and neuronal differentiation of P19 neurons. (A) protein samples of brain homogenates from mice at different age, were separated in 8% SDS-PAGE gell and examined for the presence of Dcp1a protein (E15 – 15th embryonic day, NB – newborn, 1W – 1 week, 7W – 7 weeks, 9M – 9 months). Phosphatase treatment of E15 homogenate, prior to Western-blot analysis using Dcp1a antibody. (B) Protein samples of lysates from non-differentiated (ND) and differentiated P19 neurons (D2–D8, day 2–8 post RA application), were examined by using Dcp1a antibody, and actin antibody as control.

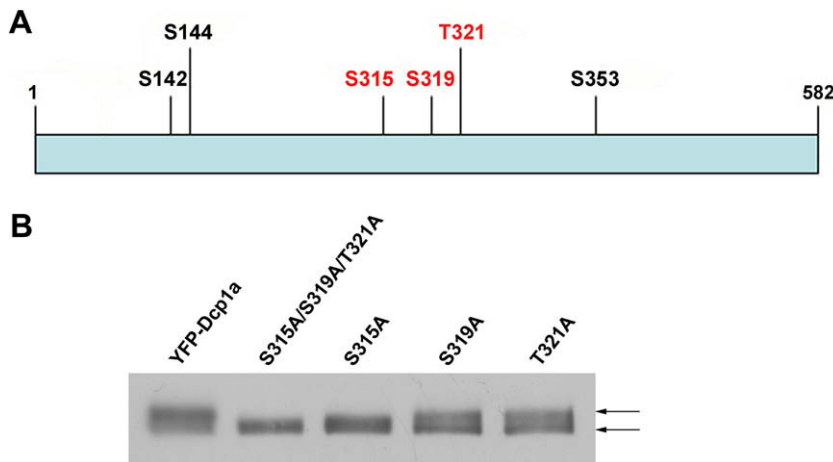
protein samples taken from several time-points along P19 neuronal differentiation. Dcp1a migrated as two distinct bands after 2 days of RA treatment (D2), similarly to its expression in non-differentiated P19 cells (ND). After 4 days of RA treatment (D4) the lower band disappeared and Dcp1a migrated as one upper band (compared to ND and D2 samples) (Fig. 1B). These results show a correlation between neuronal development stage and the phosphorylation level of Dcp1a protein.

3.2. Arsenate treatment induces P-bodies disassembly and Dcp1a phosphorylation

We followed our results revealing Dcp1a phosphorylation and monitored Dcp1a expression in response to sodium arsenate. Sodium arsenate is a toxic agent which inhibits glycolysis and induces a cellular stress response [15,16]. This agent is commonly used in order to study the effect of cellular stress and the assembly



**Fig. 2.** Arsenate induces P-bodies disassembly and Dcp1a phosphorylation in 293 HEK cells. (A) Fluorescence microscopy analysis using Dcp1a and TIAR antibodies of control and arsenate treated 293 HEK cells, scale bar = 10 μm. (B) Protein samples from 293 HEK cells of control and arsenate treated cells were separated in 8% SDS–PAGE gel and examined for the presence of Dcp1a protein. Phosphatase treatment of protein samples from arsenate treated cells were similarly analyzed.



**Fig. 3.** Dcp1a mutations expression analysis using SDS–PAGE. (A) Schematic representation of phosphorylation sites as predicted by the PHOSIDA database. (B) SDS–PAGE analysis of Dcp1a mutations as compared to the WT YFP-Dcp1a vector. Arrows mark the double bands.

of stress granules [12]. We treated 293 HEK cells with sodium arsenate (1 mM 30 min) and visualized the expression of Dcp1a and TIAR (TIA-1 related protein) as a stress granules marker. In control cells Dcp1a is localized in few large P-bodies in the cytoplasm and TIAR is found mainly in the cytoplasm and in the nucleus of the cells. Upon arsenate treatment, P-bodies are disrupted and Dcp1a localizes in smaller granules in the cytoplasm. TIAR protein, on the other hand, is found as part of large cytoplasmic stress granules (Fig. 2A). Please note that there is no colocalization between Dcp1a containing granules and stress granules. In addition, arsenate treatment induced hyperphosphorylation of Dcp1a protein in 293 HEK cells (Fig. 2B). This observation was verified by incubating protein samples of arsenate treated cells with phosphatase prior to SDS-PAGE analysis. Arsenate may induce additional post-translational modifications of Dcp1a, since an upper band of Dcp1a protein is still visible after phosphatase treatment.

### 3.3. Identification of phosphorylated residues of Dcp1a

In order to identify Dcp1a amino acid residues that are putative phosphorylation sites, we combined both bioinformatics tools and site-directed mutagenesis. We searched the PHOSIDA-phosphorylation site database at <http://www.phosida.com/> [17] for phosphorylation sites of the hDcp1a (Swiss-Prot entry: Q9NPI6). The database search predicted six possible phosphorylation sites on either serine or threonine amino acid residues, as described in Fig. 3A. We performed site-directed mutagenesis on hDcp1a sequence (cloned in a YFP vector), and mutated each residue to an alanine amino acid. We monitored the expression of YFP-Dcp1a and the mutants and found that mutations of S142, S144 and S353 had no effect on the protein migration as compared to YFP-Dcp1a (data not shown). When we followed the expression of S315, S319 and T321 mutants, we noticed that mutations of S319 and T321 amino acids migrated as double bands, whereas S315 mutation appeared as a single lower band (Fig. 3B). Interestingly, a triple mutation on S315/S319/T321 amino acids migrated as an

even lower single band compared to the migration of the S315 mutant.

### 3.4. Arsenate effect on Dcp1a mutations

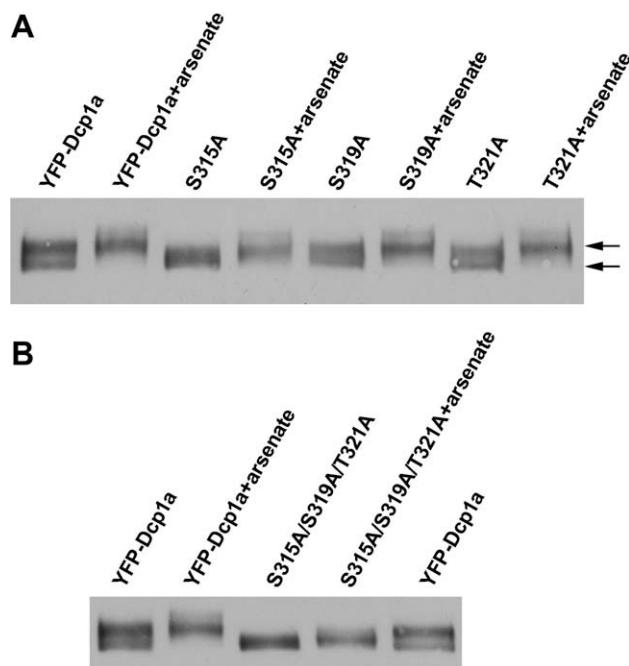
Following our previous results demonstrating that sodium arsenate induces hyperphosphorylation of Dcp1a, we analyzed its effect on the phosphorylation of Dcp1a mutants. We expressed YFP-Dcp1a plasmids harboring each of the mutations in 293 HEK cells prior to arsenate treatment of the cells. We monitored the mutations expression following arsenate treatment. Sodium arsenate induced a shift in the migration of YFP-Dcp1a and the single mutations towards an upper band (Fig. 4A). The migration patterns of the mutant proteins and YFP-Dcp1a WT plasmid following arsenate treatment are similar. However, sodium arsenate treatment induced only a minor upward shift in the band of the triple mutated protein compared to its robust effect on the WT YFP-Dcp1a protein (Fig. 4B).

## 4. Discussion

Dcp1a is a key component in the processes of mRNA decapping and degradation. Although these processes were thoroughly studied, the functional regulation of Dcp1a protein is poorly understood. In this study we examined the expression of Dcp1a protein under various physiological conditions. We found that during brain development, Dcp1a migrated as two distinct bands (Fig. 1A). The upper band was a phosphorylated form of Dcp1a, as was determined by phosphatase treatment. This observation correlates well with previous data suggesting that Dcp1p, the yeast homologue of Dcp1a, is a phosphoprotein [7]. In order to further investigate Dcp1a in neurons, we monitored its expression during P19 EC neuronal differentiation. In this model system, Dcp1a expression revealed a similar pattern of two distinct bands in the first 48 h post RA application. However, after 48 h the expression of the lower band was significantly reduced and only the upper band was observed (Fig. 1B). Phosphorylation of Dcp1a suggests that Dcp1a is part of a signaling pathway, which is activated during neuronal and brain development.

Following our previous results, we had speculated that Dcp1a could undergo phosphorylation upon other stimulations. We monitored the effect of sodium arsenate treatment of 293 HEK cells on Dcp1a expression. Sodium arsenate is a known potent toxin that inhibits glycolysis and promotes the formation of stress granules [12,15,16]. Using fluorescence microscopy we found that application of arsenate induced both disassembly of Dcp1a granules and formation of stress granules (Fig. 2A). In addition, Dcp1a in protein samples taken from arsenate treated cells migrated on SDS-PAGE as a single upper band (as compared to control samples). The expression of Dcp1a as a single upper band is partially due to phosphorylation modifications of the protein as verified by phosphatase treatment of the sample (Fig. 2B). We show here that Dcp1a phosphorylation can be induced by activation of cellular stress mechanisms. In addition, there might be a correlation between arsenate effects on P-body structure and Dcp1a hyperphosphorylation.

In order to identify phosphorylated amino acid residues of Dcp1a, we utilized bioinformatics tools. By using the PHOSIDA database combined with site-directed mutagenesis, we were able to identify 3 amino acids in Dcp1a sequence as possible phosphorylation sites (Fig. 3A). When all three were mutated (S315, S319, T321), the mutated protein migrated as a single lower band. When single mutations were introduced and analyzed, only the S315 mutant migrated as a single band, higher than the triple mutant protein (Fig. 3B). These results suggest that all three amino acids are necessary for Dcp1a phosphorylation in 293 HEK cells.



**Fig. 4.** Arsenate effect on Dcp1a mutations. Two-hundred and ninety three HEK cells were transfected with either control YFP-Dcp1a vector or mutated Dcp1a protein followed by sodium arsenate treatment. (A) SDS-PAGE analysis of Dcp1a mutations following arsenate treatment. (B) SDS-PAGE analysis of the triple mutated Dcp1a protein as compare to YFP-Dcp1a control.

We further analyzed the effect of arsenate treatment on each of the mutations. We expressed each of the mutations in 293 HEK cells prior to arsenate treatment followed by SDS–PAGE analysis. Our results demonstrate that each of the mutations migrated as a single upper band in response to arsenate treatment (Fig. 4A). Interestingly, arsenate treatment induced only a minor shift in the triple mutant protein, as compared to the shift of YFP-Dcp1a protein (Fig. 4B). From these observations we learn that arsenate can either promote Dcp1a phosphorylation on other amino acid residues than the ones we had mutated, or other post-translational modifications are involved.

In this study we examined the expression of Dcp1a protein during neuronal development and under stress conditions. Dcp1a is a key component in the mRNA decapping and degradation machinery and was previously shown to take part in the SMAD4-TGF- $\beta$  pathway. We show that Dcp1a is subjected to phosphorylation events along neuronal development and in response to arsenate-induced cellular stress. Using bioinformatics, we identified three amino acids in Dcp1a sequence, that are phosphorylated under these conditions. Taken together, our results suggest that physiological changes may influence RNA regulation via post-translational modifications of Dcp1a.

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